

Research Article

Phototrophic Growth and Accumulation of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by Purple Nonsulfur Bacterium *Rhodospseudomonas palustris* SP5212

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The ability of the phototrophic bacterium *Rhodospseudomonas palustris* SP5212 to produce polyhydroxyalkanoates (PHAs), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] in particular was, assessed in acetate medium supplemented with hydroxybutyrate and valerate as cosubstrates. The isolate accumulated the polymer accounting for some 49.06% and 30% of cell dry weight when grown in hydroxybutyrate and valerate, respectively. PHA accumulation as well as 3HV monomer incorporation (30 mol%) was maximum at 0.1% hydroxybutyrate, while valerate at 0.1% and 0.3% was suitable for total polymer accumulation and 3HV monomer incorporation, respectively. Cosupplementation of hydroxybutyrate and valerate in the ratio of 3 : 1 led to the accumulation of PHA accounting for 54% of cell dry weight, which contained more than 50 mol% of 3HV monomer. Moreover, the biphasic cultivation conditions with hydroxybutyrate as cosubstrate have improved the quality as well as quantity of the accumulated copolymer significantly.

1. Introduction

Polyhydroxyalkanoates (PHAs) represent a unique class of environmentally biodegradable polymers of commercial importance. They are synthesized and accumulated as intracellular carbon and energy rich reserve materials by a wide variety of bacteria when grown under conditions of carbon excess and at least one of the nutrients becomes limiting [1, 2]. PHAs serve as a sink for reducing equivalents depending on the organisms and the physiological conditions of the cells. Accumulated PHAs also play a significant role in the survival of the producer microorganisms under conditions of environmental stress such as osmotic pressure, desiccation, and UV-irradiation [3]. Moreover, they have received increased attention as alternatives to conventional hydrocarbon based

thermoplastics mainly because of their material properties, biodegradability, and biocompatibility.

The incorporation of 3-hydroxyvalerate (3HV monomer) into the polymer of 3-hydroxybutyrate (3HB) can result in the synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] with improved thermoplastic properties that are more suitable for commercial application [4]. It is, therefore, apparent that the PHA materials can be tailored to specific applications by varying their chemical structure [5], which could be achieved by variation of producing organisms, carbon sources, cosubstrates and cultural conditions.

Altogether, 150 hydroxyalkanoic acids have been identified as constituents of PHAs [6], and these subunits of PHA have been broadly subdivided as short chain length (C3–C5) and medium chain length (C6–C14) monomers.

PHAs composed of different molar proportions of these monomers differ markedly in terms of their physicochemical, mechanical, and thermal properties [7].

Quantitative studies on PHA metabolism by phototrophic purple nonsulfur bacteria are restricted only to a few species like *Rhodospirillum rubrum* [3], *Rhodobacter sphaeroides* [8–12], and *Rhodopseudomonas palustris* [13, 14]. *R. palustris*, the widely known species of purple non sulfur bacteria, accumulate P(3HB) [13, 15] but only to a limited scale. Vincenzini et al. [16] have reported that *R. palustris* 420l accumulate P(3HB) of nearly 18% of cell dry weight (CDW) when grown in phosphate limiting condition in a biphasic growth mode. The same isolate, in a temperature controlled underwater tubular photobioreactor in outdoor conditions, produces the highest amount of P(3HB) (4.0% of CDW) in summer in comparison to its lowest accumulation (1.1% of CDW) during winter. *R. palustris* cells when grown diazotrophically with acetate were reported to accumulate 1.1% to 0.3% of CDW of P(3HB), but under nitrogen fixing condition they are known to accumulate 8.8% P(3HB) of CDW after exhaustion of ammonium from the medium. The species along with *Blastochloris sulfoviridis* accumulate P(3HB) accounting for 1.4–3.6% of CDW in a lighted upflow anaerobic sludge blanket (LUSAB) [17].

Very little was known about the potential incorporation of monomer units other than 3-hydroxybutyrate into the polymer, leading to the formation of various 3-hydroxyalkanoate copolymers by this particular species. *R. palustris* 1850 and 17001 incorporated 3HV monomer unit in the polymer when grown with valerate, heptanoate, and octanoate. However, strain 1850 was unable to incorporate 3HV when grown with propionate [9]. *R. palustris* SP5212 isolated in this laboratory has been reported to accumulate P(3HB) accounting for 15.0% of cell dry weight with acetate as substrate and was capable of utilizing C2–C8 alkanolic acids for growth and polymer production [14]. The objective of the present study was to evaluate the synthesis of copolymers of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *R. palustris* SP5212 and to optimize the cultural conditions for such PHA accumulation. A biphasic cultivation method with alkanolic acid as cosubstrate has also been implemented for qualitative and quantitative improvement of the accumulated polymer.

2. Materials and Methods

2.1. Bacterial Strain and Cultural Condition. *Rhodopseudomonas palustris* SP5212 reported from this laboratory [14] was used throughout this study. The isolate was grown in 15 mL screw cap bottles under microaerobic phototrophic conditions using malate medium (g/L, malic acid, 0.2; K_2HPO_4 , 0.9; KH_2PO_4 , 0.6; $MgCl_2$, 0.2; nicotinic acid, 0.005, EDTA, 0.02; NH_4Cl , 1.0; yeast extract, 1.0 (pH 6.8)). The same medium with minor modifications was used for PHA accumulation. Malate was replaced by acetate as the main carbon source, ammonium chloride was omitted to induce nutritional stress, and alkanolic acids were supplemented as the cosubstrate. Experiments were conducted in 300 mL

transparent glass stoppard bottles. The medium was inoculated with a freshly grown culture (O.D. 0.5 at 650 nm) at 2.0% (v/v) level and incubated under microaerophilic condition with a continuous illumination (10,000 lux) at 30°C.

For biphasic growth experiments, *R. palustris* SP5212 was initially grown in malate medium for 4 days, and cells were harvested aseptically, washed thoroughly with sterile 0.9% NaCl, and transferred to a similar 300 mL transparent glass stoppard bottle containing acetate medium. The medium was supplemented with alkanolic acids and incubated under continuous illumination (10,000 lux) at 30°C.

2.2. Determination of Growth. Growth of the organism was determined by measuring the dry weight of the biomass. Cells were harvested by centrifugation at 12,500 ×g in a Hitachi SCR 20B centrifuge, washed thoroughly, transferred to preweighed aluminium cups, and dried to a constant mass at 80°C.

2.3. Analysis of Polyesters. The polymer was extracted from the dried cell mass directly with boiling chloroform [19], concentrated and precipitated with double volume of prechilled diethyl ether, separated by centrifugation (12,000 ×g for 10 min), and dried under vacuum at room temperature. For purification, the polyester was redissolved in chloroform, filtered and further precipitated with chilled diethyl ether, and dried under reduced pressure. The amount of PHA was quantified gravimetrically and expressed as the percentage of cellular dry weight [18]. Monomer composition of the PHAs was determined by 1H NMR spectroscopic analysis. The purified polymer, dissolved in analytical grade deuteriochloroform ($CDCl_3$), was subjected to analysis, and the chemical shifts were recorded using a Bruker AMX 300 NMR spectrophotometer with a multinucleate probe head.

3. Results and Discussions

Time course of growth and polymer accumulation by *R. palustris* SP5212 under phototrophic conditions in the acetate medium supplemented with hydroxybutyric acid (0.1% w/v) as cosubstrate revealed gradual increase in growth and polymer accumulation with the period of incubation. The accumulated polymer attained its maxima after 12 days of incubation (Figure 1(a)) when the PHA content reached 49.06% of cell dry weight (CDW). It was evident that during the early stages of growth there was no incorporation of 3-hydroxyvalerate (3HV) in the polymer but with time the 3HV monomer unit incorporation in the P(3HB-co-3HV) increased up to 37 mol% after 10 days of growth (Figure 1(b)). Similarly, supplementation of valeric acid as cosubstrate also revealed similar pattern of growth and 3HV incorporation in the polymer (Figure 2(a)). At 12 days of growth the accumulated PHA (30% CDW) of *R. palustris* cells contained nearly 14 mol% of 3HV monomer unit (Figure 2(b)). The present experimental results have clearly established the synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] by *R. palustris* SP5212 utilizing hydroxybutyrate as well

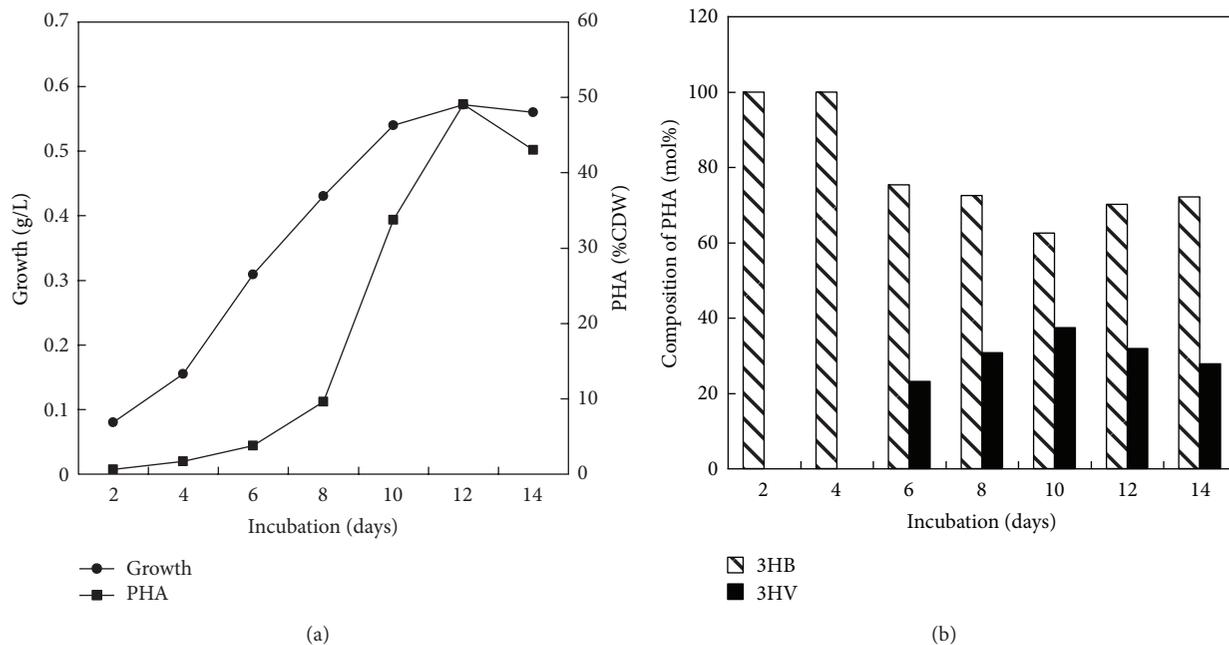


FIGURE 1: Time course of growth, PHA content (a), and composition (b) of the polyester accumulated by *R. palustris* SP5212 under phototrophic conditions in acetate medium supplemented with hydroxybutyrate as cosubstrate.

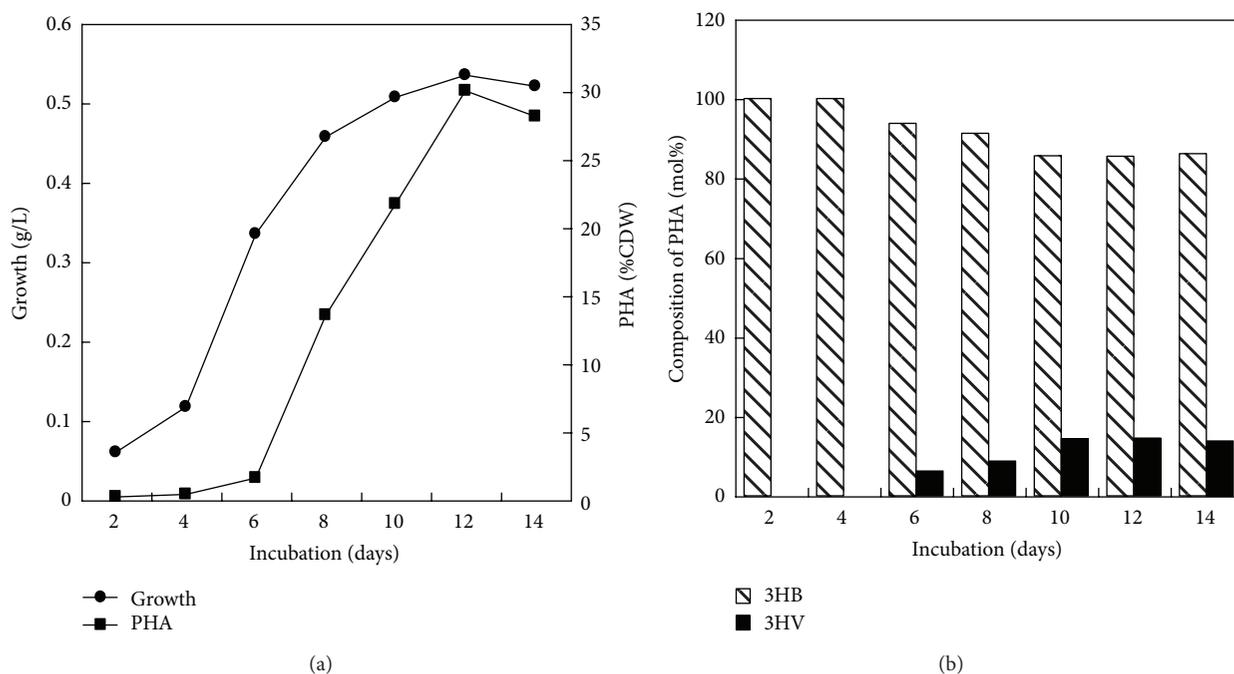


FIGURE 2: Time course of growth, PHA content (a), and composition (b) of the polyester accumulated by *R. palustris* SP5212 under phototrophic conditions in acetate medium supplemented with valerate as cosubstrate.

as valerate as cosubstrate and acetate as the principal source of carbon. Most of the earlier studies with *R. palustris* reported accumulation of only P(3HB) under different cultural conditions [13, 15, 17]. However, accumulation of copolymers containing C4-C5 monomers was reported only when

the organism was grown on alkanolic acids [9, 14]. *Rhodospseudomonas acidophila* accumulated polymer with 91.0 mol% of 3HV when grown with C5 compounds [9]. In contrast, *Rhodospirillum rubrum* accumulated copolymers of C4, C5, and C6 compounds with higher carbon substances [3].

TABLE 1: Effect of hydroxybutyrate and valerate on growth and PHA accumulation by *R. palustris* SP5212.

Concentration, % (w/v)	Growth, g/L	PHA, % CDW	Composition of PHA, Mol%	
			3HB	3HV
Hydroxybutyrate				
0.05	0.47	23.25	73.70	26.30
0.10	0.57	49.06	70.19	29.80
0.20	0.49	36.33	77.18	22.82
0.30	0.41	16.78	81.98	18.01
Valerate				
0.05	0.52	15.19	87.44	12.56
0.1	0.50	29.84	85.64	14.36
0.2	0.44	20.31	71.13	28.87
0.3	0.39	17.43	62.39	37.61

The isolate was grown in acetate medium supplemented with hydroxybutyrate and valerate as cosubstrate under microaerophilic condition for 12 days at a light intensity of 10,000 lux, total PHA content was estimated according to the method of Ulmer et al. (1994) [18], and growth was determined by biomass dry weight.

The influence of different concentrations of hydroxybutyrate and valerate on growth and polymer synthesis by *R. palustris* was tested under identical cultural conditions, and the optimum concentration of hydroxybutyrate and valerate for synthesis of PHA was found to be 0.1% (w/v). However, the PHA content differed significantly and was 49.06% and 29.8% CDW for hydroxybutyrate and valerate grown cells respectively. The 3HV monomer unit incorporation was maximum (29.8 mol%) with 0.1% of hydroxybutyrate, while with valerate, the 3HV content increased from 12.56 to 37.6 mol% with increasing concentration (0.05–0.3% w/v) of the cosubstrate used (Table 1). It was evident that the polymer accumulation efficiency and the monomeric composition of the accumulated PHAs by *R. palustris* SP5212 were correlated with the concentrations of cosubstrate supplemented in the growth medium. Moreover, the strain was able to direct more 3HV monomer (37 mol%) to copolymer with valerate compared to hydroxybutyrate (Table 1). Mutant strain of *Rhodobacter sphaeroides* U7 when grown in cosubstrate acetate-valerate (40/40 molar ratio) shows the highest accumulation of copolymer (77.42%) with 84.77% fraction of hydroxyvalerate [12].

The effect of combined supplementation of hydroxybutyrate and valerate in different proportion during the initiation of growth of *R. palustris* in acetate medium resulted in significant increase in both polymer accumulation and 3HV monomer incorporation in the synthesized PHAs (Table 2). Maximum polymer accumulation (54.0% CDW) was achieved when hydroxybutyrate:valerate ratio was maintained at 3:1. The ^1H spectra of this accumulated copolymer showed the incorporation of more than 50 mol% of 3HV monomer unit (Figure 3). Previously, ^1H NMR study was employed to follow the process of accumulation and decomposition of P(3HB) in *R. palustris* [20]. The ^1H NMR spectral analysis revealed that 3HV monomer content of

TABLE 2: Effect of cosupplementation of hydroxybutyrate and valerate on growth and PHA accumulation by *R. palustris* SP5212.

Hydroxybutyrate:Valerate	Growth, g/L	PHA, % CDW	Composition of PHA, Mol%	
			3HB	3HV
1:3	0.49	26.77	57.66	42.33
1:1	0.52	46.26	66.61	33.38
3:1	0.66	54.71	48.47	51.52

The isolate was grown in acetate medium supplemented with hydroxybutyrate and valerate in different proportion, and other conditions are the same as mentioned under Table 1.

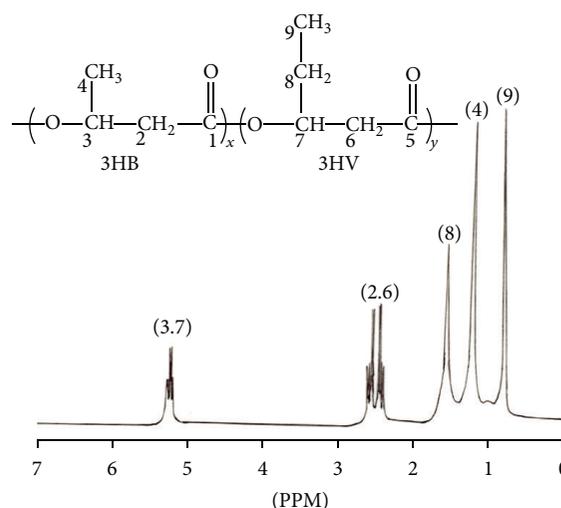


FIGURE 3: ^1H NMR spectra of polymer extracted from *R. palustris* SP5212 cells grown under phototrophic conditions in acetate medium supplemented with mixtures of hydroxybutyrate:valerate (3:1).

the polymer was increased further (51 mol%) when mixtures of both alkanolic acids were used at 3:1 ratio. The intracellular level of the polymer also rose to more than 54% of CDW (Table 2).

Finally, attempts were made to grow *R. palustris* SP5212 in biphasic cultivation condition. The organism was initially grown in malate medium for 4 days under phototrophic, microaerobic conditions; cells were harvested aseptically by centrifugation (12,000 \times g), washed in sterile saline solution, and immediately transferred to acetate medium supplemented with either hydroxybutyrate or valerate as cosubstrate. A significant increase in polymer accumulation was recorded under both conditions (Table 3). The PHA content attained 54% and 37% of cell dry weight when grown with hydroxybutyrate and valerate, respectively. Compositional analysis revealed that PHAs derived from hydroxybutyrate and valerate were copolymers of 66% 3HB-co-34% 3HV and 69% 3HB-co-31% 3HV, respectively. These results corroborate the findings of Vincenzini et al. [16], who also recommended two-step cultivation for improved polyester accumulation in *R. palustris*. Increase in polymer content from 3% to 18% of CDW was achieved when biphasic process was imposed in

TABLE 3: Biphasic growth and PHA accumulation by *R. palustris* SP5212 with hydroxybutyrate and valerate as cosubstrate.

Cosubstrate	Incubation, days	Growth, g/L	PHA, % CDW	Composition of PHA, Mol%	
				3HB	3HV
Hydroxybutyrate	6	0.42	11.26	72.57	27.42
	8	0.50	29.13	68.67	31.32
	10	0.61	54.17	66.59	33.41
	12	0.49	39.47	65.07	34.92
	14	0.45	37.34	57.17	42.82
Valerate	6	0.33	14.22	76.15	23.84
	8	0.38	23.20	74.05	25.94
	10	0.45	37.40	69.13	30.86
	12	0.51	29.16	67.03	32.96
	14	0.49	25.16	63.96	36.03

The isolate was grown first in malate medium for 4 days, and cells were harvested, washed, and aseptically transferred to acetate media supplemented with 0.1% hydroxybutyrate and 0.1% valerate; other conditions are the same as under Table 1.

this particular strain. *Rhodobacter sphaeroides* 14F showed 3.5 g/L PHA with 60% PHA content when grown in two-stage aerobic dark cultivation at 37–40°C [10].

4. Conclusion

The potential of *R. palustris* SP5212 to produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate) under phototrophic microaerophilic conditions has been assessed in acetate medium supplemented with alkanolic acids as cosubstrates. Cosupplementation of mixtures of hydroxybutyrate and valerate as well as biphasic cultivation conditions has improved the PHA accumulation efficiency of the strain significantly, which indicates possible utilization of *R. palustris* SP5212 for large scale production of PHA copolymers with improved material properties.

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